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Molecular evaluation of microalgal communities in full-scale waste stabilization ponds

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Molecular evaluation of microalgal communities in full-scale waste stabilization ponds

Waste stabilization ponds (WSPs) are widely used across the world as a passive wastewater treatment for domestic wastewaters, but little is known about their ecology, especially their phototrophic communities. This study uses molecular methods and flow cytometry to assess the cyanobacterial and eukaryotic communities longitudinally throughout two systems, one treating domestic wastewater and the other mixed industrial/domestic wastewaters. More variation was seen between the systems than between different stages in the treatment processes for both eukaryotic and cyanobacterial communities. *Chlorella* species and *Planktophrix* cyanobacteria dominated both treatment systems. *Arthrospira* cyanobacteria were detected only in the industrial/domestic system. The balance between non-photosynthetic and photosynthetic organisms is rarely considered, though both play vital roles in WSP functioning. Flow cytometry showed that the facultative and first maturation pond in the industrial system contained a lower proportion of photosynthetic organisms compared to the domestic system. This is reflected in the species richness data, and the low dissolved oxygen levels detected. All data indicated that the two systems are significantly different from one another and that variation longitudinally throughout the systems is lower. A more systematic study is determine if it is the wastewater source rather than the initial inoculum that drives community composition.

Keywords: ecology; microalgae; molecular microbiology; waste stabilisation ponds, wastewater, phytoplankton

Introduction

As countries develop and rapid expansion of industry occurs, processes that can effectively treat industrial wastewater are required to minimize environmental damage. Waste Stabilization Pond (WSPs) systems have long been used for domestic wastewater treatment [1]. WSP systems treating a mixture of domestic and industrial wastewaters are becoming more common, especially in countries whose industrial sectors are rapidly expanding.

WSPs are widely used across the world as a passive wastewater treatment technology, but are often treated as a 'black box' with little known about their ecology, especially their phototrophic communities. Currently, systems are designed empirically, based on organic loading [2]. A better understanding of the biological processes involved in treatment may help to optimise designs. Integral to WSP systems are microorganisms, including non-photosynthetic bacteria (both anaerobic and aerobic, depending upon pond conditions), photosynthetic bacteria and photosynthetic eukaryotes [1]. Photosynthetic organisms are believed to make up the vast majority of biomass in facultative ponds, and especially in maturation ponds that usually present conditions that do not limit light penetration. However, this has yet to be quantitatively evaluated and verified. Bacteria and microalgal interactions can range from symbiotic to parasitic [3]. Cycling of oxygen and carbon dioxide between photosynthetic microalgae and aerobic bacteria result in a mixed community of both organism groups. The efficiency of wastewater treatment processes in WSPs depends on the balance between these two groups of organisms.

Current knowledge of WSP ecology is based predominantly on taxonomic microscopy studies [4-6], which are slow and rely heavily on the expertise and training of taxonomic specialists. Microscopy techniques are also limited by image resolution and reliance on the morphology of organisms being investigated being distinct. Different sample preparation methods and staining are required to identify different cellular features making a one-size fits all approach to a complex sample difficult. The use of molecular biology techniques, commonly used to study bacterial communities [7, 8], is in its infancy for the investigation of eukaryotic and prokaryotic phototrophs in WSPs. A number of studies have assessed the bacterial populations [10, 11] and functional groups such as nitrogen processing organisms[12].

Ghosh and colleagues [9] presented an assessment of microalgae and cyanobacteria in wastewater polishing ponds using two sets of primers targeting different forms of the RuBisCO gene (rbcL) and a clone library approach. This approach was able to detect greater species diversity than has been previously estimated by microscopy studies. A more recent study, by Wallace and colleagues [13], successfully used Ion Torrent sequencing to target eukaryotic microalgae in three waste stabilization ponds in Canada. They focused on photosynthetic eukaryotes only and did not assess cyanobacteria, which have the potential to produce toxins and affect nutrient cycling in ponds.

The aim of the current study was to assess, using molecular methods, the communities of photosynthetic organisms (including cyanobacteria and photosynthetic eukaryotes) in two stabilisation pond systems treating wastewater in the tropics (Brazilian northeast).

Methods

Sampling sites

The Ceará State Company for Water and Wastewater (CAGECE) allowed sampling in two full-scale waste stabilization pond systems: the Marechal Rondon system (MR), which treats solely domestic wastewater, and the Sistema Integrado do Distrito Industrial (SIDI), at Maracanaú, which treats effluents from the textile and leather industries as well as domestic wastewater. SIDI wastewater often contains a mixture of inorganic and organic compounds including dyes, dying aids and sizing agents from the textile industry and tanins, chlorides, proteins, non-ionic surfactants and oils from the tanneries [14, 15].

Manufacturers of metal products, plastics, concrete and ceramics, food products, soft drinks, PVC and cardboard also contribute to the SIDI wastewater stream, as do two industrial scale laundries and a poultry abattoir. The wastewater stream is mixed at a ratio of 1:1 with domestic wastewater in order to supplement nutrients and encourage growth of organisms involved in the treatment process.

The MR system consists of an anaerobic (80m x 68m), two parallel facultative (approximately 86m x 284m and 77m x 198m) and two maturation ponds in series (both approximately 200m x 98m). The SIDI system contains an anaerobic (1047m x 22m x 4m), one facultative (1055m x 30m x 2m) and three maturation ponds in series (1052m x 155m x 1.5m, each). The pond systems were designed for a median flow rate of 280 l/s (max. of 520 l/s) for SIDI and 76.6 l/s for MR (max. of 132 l/s). Hydraulic retention times were estimated to be 9 days (facultative) and 5.5 days (maturation) for SIDI, and 5.5-7.5 days (facultative 1), 3.5-4.5 days (facultative 2) and 3-4.5 days (maturation) in MR. These retention times are based on design dimensions and current flow rates. Actual retention times may be shorter due to sludge accumulation in the ponds. A tracer study would be required to determine actual retention times accurately, though this was outside of the scope of this study. Figure 1 shows the location of all sampling points in both systems.

Grab samples for DNA extractions were collected from two sampling points per pond in the two systems, one close to the influent and another close to the effluent. Samples were taken 5 cm below the surface of ponds in sterile 1 litre Duran bottles and transported on ice to the laboratory. Aliquots of 5mls of each sample was fixed overnight with 2% paraformaldehyde (PFA) and stored in 50:50 PBS and ethanol at -20’C, for flow cytometry. Collection took place in February 2011 at the start of the wet

season. Another set of samples were collected at the inlet of the treatment plant and at the outlet of each of the ponds for physicochemical analysis, see Table 1.

Physicochemical analyses

A multiparameter probe was used to measure pH of field samples. Samples were collected and taken back to CAGECE's laboratory for Total suspended solids (TSS), Total solids (TS) by gravimetry analyses. Biochemical oxygen demand (BOD), Chemical oxygen demand (COD), and dissolved oxygen were measured with titrimetry. Total coliforms and *E.coli* counts were performed using a chromatographic assay. Nitrate and nitrite concentrations were measured using colorimetric spectrophotometry methods and orthophosphate using the ascorbic-acid method. Ammonia was measured using the ammonia-selective electrode method. All these analyses were performed following standard methods [16].

Cell counting

PFA fixed samples were used for flow cytometry. Phototrophic and non-phototrophic fractions were distinguished and counted using a FACs ARIA flow cytometer (Flow Cytometry Core Facility, Newcastle University) on the basis of the fluorescence of photosynthetic pigments [17]. Side scatter and the 488/710/50 laser and detectors were used. Activated sludge was used as a negative control and several pure algal cultures as positive controls. The full method development and evaluation are described in detail by [18].

DNA extraction and PCR amplification

DNA was extracted from 80ml samples using a DNeasy® Blood and Tissue kit (Qiagen, UK), following the manufacturers protocol, as previously described [19].

Amplification of 18S and 16S gene fragments was carried out in duplicate by PCR. A GC clamp was added to the 5'-end of the forward primers for PCR for DGGE analysis. PCR was carried out using three primer sets Euk1A and Euk 516r [20] to target 18S rRNA genes in eukaryotic microalgae and Cya-b-F371 and Cya-R783 and modified 2/3 [21] called F357GC and R518 to target cyanobacterial 16S rRNA. Cya-b-F371 and Cya-R783 amplification product was nested with the F357GC and R518 primers [22]. PCR was carried out using PCR MegaMix Blue® (Microzone, UK), 1µl of each primer and 1µl of DNA extract using a BioRad C1000™ thermocycler. PCR reaction cycles and conditions used can be seen in primer papers.

Community analysis

The predominant eukaryotic and cyanobacterial organisms within the communities were compared using DGGE (BioRad system). Gradients were optimised for each primer set. Electrophoresis was run on 0.75mm thick, polyacrylamide gels (37.5:1 acrylamide:bisacrylamide). For PCR products amplified with Eukaryotic primers, a 6% gel with linear gradient of denaturing agents from 15% to 40% (where 100% denaturing agent is defined as 7mol L-1 urea and 40% deionized formamide) was used. For cyanobacterial nested PCR products, an 8% gel and a denaturing gradient of 20% to 60% was used. Gels were stained using Sybr Gold DNA dye.

Dominant bands were excised from DGGE gels for sequencing. DNA was amplified from cut bands using the initial primers and PCR programs. A QIAquick PCR purification Kit (Qiagen, UK) was used to clean up DNA before Sanger sequencing (GeneVision, Newcastle, UK). Sequences were aligned and compared using NCBI BLAST [23] against the nucleotide collection (nr/nt) databases and cyanobacterial sequences were entered into the RDP classifier [24].

BioNumerics (Applied Maths, Belgium) was used to normalise bands within the DGGE gel and to perform cluster analysis. Gene copy numbers in eukaryotes are more variable than those in prokaryotes [25], so band intensity may be skewed by species with higher copy numbers. To reduce this bias, band presence/absence data were used in Primer 6 software [26] to analyse the similarity between samples for both communities.

Species richness (S) was calculated using presence/absence data from normalised and Pieloi's evenness index (J') using band height data from normalised DGGE gel images. Each band was deemed to represent a unique operational taxonomic unit (OTU). Pielou's evenness index is a measure of equitability and was calculated using Equation (1) below, where H'_{max} is the maximum possible value of Shannon Wiener diversity (H').

$$J' = \frac{H'}{H'max} = \frac{H'}{logS} \qquad (1)$$

MDS (Multidimensional Scaling) analysis was carried out and ordination plots produced using presence/absence data generated from the DGGE images. Two-way crossed ANOSIM (Analysis of Similarity) was used to assess the differences between samples from the two sites and between samples from different stages in the treatment systems, for both eukaryotic and cyanobacterial community data.

Results and discussions

Treatment performance

The wastewater influent in SIDI is characterised by low ammonia concentrations (10.47mg N-NH_3/l) and high solids (1852 mg/l), whilst the influent to the MR system has comparatively higher ammonia (51.68 mg N-NH_3/l) and phosphate levels (6.421 mg P-PO_4^{-3}/L) (Table 1). For both treatment systems, a three log reduction in faecal

coliforms was achieved (99.99 %). Total suspended solids also decreased in both SIDI and Marechal Rondon, by 70% and 81.2% respectively. The starting BOD was not supplied for the SIDI treatment system, but COD concentrations decreased by 66%, throughout the system, with the biggest reduction occurring in the anaerobic pond. COD reduction of 76% and BOD reduction of 89% were achieved by the domestic system (MR). The data show that the MR system effectively removed ammonia, with a reduction of 83%, but was less efficient at phosphate removal (42.21% decrease). The SIDI treatment data suggest the opposite, with a 15% increase in ammonia and a 96% reduction in orthophosphate concentrations. Low levels of oxygen were measured throughout the SIDI treatment process.

Cell counts

The SIDI treatment system had overall higher non-photosynthetic community proportions than the MR system (Figure 2). This result was supported by visual assessment of the ponds, with MR ponds appearing green and SIDI ponds first facultative and maturation ponds appearing black and pink respectively. The black colouration can be explained by the high proportion of indigo dyes that could be observed within the samples. The pink colouration may be a result of growth of purple sulphur bacteria, common in ponds with anoxic conditions and the presence of sulphides [27]. The low nutrient levels (both ammonia and orthophosphate) and the darker effluent colour in the SIDI treatment plant may account for the lower proportion of photosynthetic organisms in this system, compared to Marechal Rondon.

Community analysis

The eukaryotic communities were found to be most similar in samples taken from the same pond system; with all MR samples having a similarity of greater than 60% (Figure

3a). The eukaryotic community was more variable in the SIDI system forming two clusters. Broadly, those samples from near the entrance of the system are more dissimilar than all of the later samples and those from the MR system, with less than 40 % similarity (Figure 3a) (ANOSIM, R = 0.668, P = 0.03). This suggests that the nature of the wastewater entering the treatment system and the inoculum used to start the system may have a greater effect on the species that dominate it, rather than the type of pond (facultative vs. maturation), as shown in the ecology of activated sludge by Curtis and colleagues [28]. This may be as a result of the low nutrient levels in the SIDI wastewater input, compared to the domestic system. The pattern is similar, though slightly less pronounced, for the cyanobacterial community, with the two systems being largely dissimilar from one another (ANOSIM, R = 0.667, p = 0.037) (Figure 3b). Among treatment stage groups of samples, eukaryotic communities were more similar between treatment stages (ANOSIM, R= 0.186, sig. 20%) than between sites (ANOSIM, R = 0.833, sig. 3.7%). A more systematic study would be required to confirm whether stochastic immigration or environmental niche effects are driving community differences.

Pieloi's evenness index (J') for cyanobacteria tended to be relatively constant throughout the systems (Table 2). In the domestic system, the J' index decreased in the final pond to 0.7872. Cyanobacterial species richness (S) showed an upward trend throughout the SIDI system, ranging from 6 OTUs in the first facultative sample to 17 OTUs in the final maturation sample. This dramatic increase in cyanobacterial band richness was not seen in the MR samples, with a smaller range between 8 and 12 OTUs, the smallest value occurring in the final pond. Eukaryotic band richness showed a similar pattern, with a wide range from 3 bands to 14 in the SIDI system and only 7 to 13 in the MR system. This suggests that there is more variability in community structure

in samples across the SIDI system than the MR system. This may be linked to the more extreme chemical conditions likely present in an industrial effluent, such as the presence of dye waste and sulphur compounds (not measured) early in the treatment process. Dye waste in the facultative ponds contributed to the high total solids recorded and may be limiting light penetration in the pond, hindering the survival of algal species.

A total of nine of the dominant eukaryote DGGE bands and 17 of the dominant cyanobacterial bands were sequenced. Sequencing data showed a high eukaryotic diversity, including microalgae such as *Chlorella sorokiniana* and *Parachlorella kessleri* and ciliates such as *Opisthonecta minima* and rotifers like *Brachionus calyciflorus*. Chlorella species appear to be common across both pond systems and in all stages of the treatment. The presence of *Parachlorella* was detected in all SIDI samples, but only at low levels in the domestic system.

Planktothrix rubescens or *P.agardhii* related cyanobacterial bands were seen to be the dominant cyanobacteria in the facultative ponds of the SIDI plant and throughout all of the MR system. Bands matching *Arthrospira* in the database were found in the SIDI treatment system (particularly in the first half of the treatment process). This group of organisms is commonly found where pH and dissolved solid levels are high, as is the case in the SIDI system [1].

Sequencing of cyanobacterial bands also highlighted problems commonly seen in the molecular identification of photosynthetic prokaryotes [29]. The shared evolutionary history of cyanobacteria and eukaryotic chloroplasts, results in 16S rRNA genes being present in the chloroplasts of eukaryotes [30]. Around half of the bands seen in the DGGE had sequences whose best matches in the database were algal chloroplasts or plastids. A more detailed discussion on common bias due to the use of

molecular and taxonomic methods to study phytoplankton communities in WSP can be found in [18].

Though difference in the microalgal and cyanobacterial communities was seen between the samples from each of the treatment ponds in treatment process, the samples only represent specific locations within the system. Variation in flow rate and mixing of the wastewater, sediment build up (affecting depth) and proximity to the edge of the pond could all potentially affect the microalgae found at different locations. The depth at which sampling was carried out, also has the potential to affect the presence of different algal taxa. Motile algae (such as *Euglenophyceae* and *Chlamydomona*s) and cyanobacteria are known to adjust their position in the water column depending on light intensity, known as negative phototaxis [31].

Conclusions

The ecology of the two wastewater treatment systems have inherent differences, including the proportions of non-photosynthetic to photosynthetic organisms and also the patterns of diversity in longitudinal succession throughout the pond series. There are however some OTUs common across both systems, with sequencing showing *Chlorella* species to be the most common in both treatment pond systems.

The most significant differences in community structure are between the sets of samples from the two different pond systems, rather than those taken in different pond types. In order to establish a direct link between the community differences and the wastewater source treated, an extended study including more sites, over a longer period and accounting for initial inoculum use to seed the ponds would be required.

Further work on improving the database used for comparing 18S rRNA sequences will be required if molecular biology techniques are to become common

place in WSP research. This will require close work with taxonomic specialists to sequence microalgae commonly found in WSPs.

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Table 1. Chemical, physical and biological data for treatment processes. ND- Not detected, NP- Not provided

		Raw sewage Influent	Anaerobic output	Facultative output	Maturation 1 output	Maturation 2 output	Maturation 3 output
pH	MR	7.31	7	7.65	8.12	7.95	
	SIDI	8.56	7.62	8.09	8	7.74	7.45
Total suspended solids (mg/l)	MR	405	31	71	90	76	
	SIDI	176	110	70	85	66	52
Total solids (mg/l)	MR	1185.5	592.5	578	606.5	580.5	
	SIDI	1852	1565	1217	1321.5	1193.5	NP
Ammonia (mg N-NH_3/l)	MR	51.68	31.69	15.78	13.04	8.91	
	SIDI	10.47	13.47	14.47	11.83	12	NP
Orthophosphate (mg P-PO_4^{-3}/l)	MR	6.421	3.79	3.099	2.813	3.711	
	SIDI	2.56	1.478	0.125	0.109	0.106	NP
Nitrate (mg N-NO_3^-/l)	MR	0.027	0.07	0.056	0.085	1.608	
	SIDI	0.024	ND	ND	ND	ND	ND
Nitrite (mg N-NO_2^-/l)	MR	0.005	ND	ND	0.018	0.71	
	SIDI	ND	ND	ND	ND	ND	ND
BOD (mg O_2/l)	MR	626.6	158.15	120.36	126.12	69.19	
	SIDI	NP	NP	NP	NP	89.41	129.55
COD (mg O_2/l)	MR	704.5	194.3	174.1	194.3	170	
	SIDI	513.2	362	277	244.4	166.3	175.2
Dissolved Oxygen (mg O_2/l)	MR	NP	NP	8.7	8.5	13.4	
	SIDI	NP	NP	ND	0.19	0.79	1.9
Total coliforms (cells/100mls)	MR	9.9×10^7	2.2×10^6	1.0×10^6	5.1×10^5	8.6×10^4	
	SIDI	6.1×10^7	3.0×10^6	2.4×10^7	3.6×10^5	5.3×10^5	1.9×10^5
E.coli (cells per 100mls)	MR	2.9×10^7	9.6×10^5	1.1×10^5	9.3×10^3	2.4×10^3	
	SIDI	1.1×10^7	8.3×10^5	3.8×10^6	8.1×10^4	4.2×10^3	$<1.0 \times 10^2$

Table 2. Pieloi's evenness index (J') and Species (OTU) richness (S) for the cyanobacterial and eukaryotic communities in treatment system samples.

	SIDI cyanobacteria		SIDI eukaryotes		MR cyanobacteria		MR eukaryotes	
	J'	S	J'	S	J'	S	J'	S
F1 or F1i	0.8748	6	0.8662	3	0.8156	11	0.9002	9
F2 or F1e	0.8406	14	0.6781	9	0.8403	10	0.6497	7
M1i	0.8374	8	0.9703	4	0.8148	10	0.9598	10
M1e	0.8751	12	0.8529	3	0.8144	10	0.7912	10
M2i	0.911	14	0.7977	7	0.8292	12	0.7751	12
M2e	0.8708	12	0.7814	13	0.7872	8	0.7234	13
M3i	0.8637	12	0.8431	14				
M3e	0.8775	17	0.8706	14				

Figure 1. Satellite images (Google Earth Imagery ©10/05/2009) of the two WSP systems, SIDI on the left and Marechal Randon on the right. Arrows show the location of the inlet and outlet pipes, dots and writing show the sample collection points and names. SIDI is located at 3°51’19” S, 38°37’24” W and Marechal Randon at 3°46’44” S, 38°38’11” W.

Figure 2. Flow cytometry counts of photosynthetic and non- photosynthetic events in WSP samples. Samples named as follows; F1- Facultative pond 1, F2- Facultative pond 2, F1i- Facultative pond 1, influent, F1e- Facultative pond 1, effluent, M1i- Maturation pond 1, influent, M1e- Maturation pond 1, effluent, M2i- Maturation pond 2, influent, M2e- Maturation pond 2, effluent, M3i- Maturation pond 3, influent, M3e- Maturation pond 3, effluent.

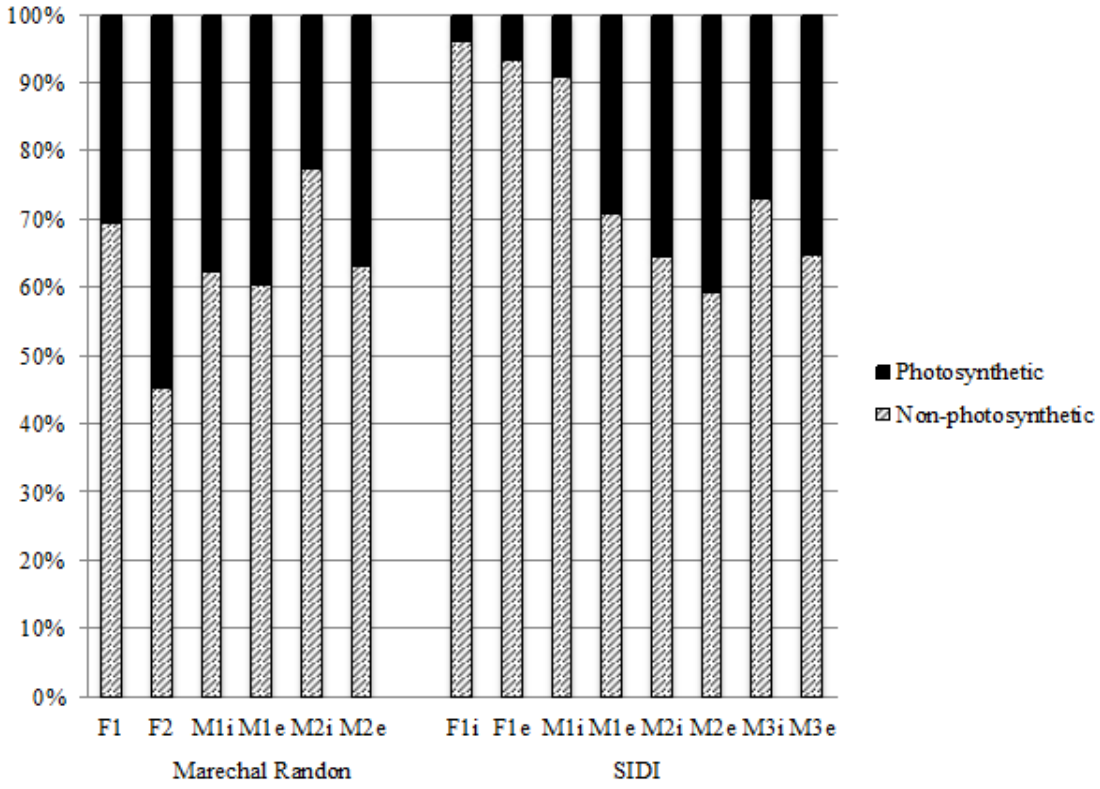

Figure 3a & b. Primer 6 Non-metric multidimensional scaling (MDS) ordination plots based on similarity between a) Eukaryotic community in samples, b) Cyanobacterial community in samples, based on DGGE data. Contours represent the degree of similarity expressed as a percentage.